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Stable suspensions of coated magnetic particles, preferably resuspendable bioactive particles particularly useful in Magnetic Resonance Imaging, are produced by disrupting, what are presumed to be, crystalline agglomerates of a parent particulate magnetic starting material in the presence of a coating material, such that coating can take place during the disruption. The particles are generally coated in suspension yielding a stable suspension of subdivided particles. With the proper selection of a coating material, preferably a protein or other biochemically or biologically active polymer such as an antibody, a resuspendable (colloidal) bioactive product is obtained.

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- 1 -

RESUSPENDABLE COATED MAGNETIC PARTICLES AND STABLE MAGNETIC
PARTICLE SUSPENSIONS

This application is a continuation-in-part of Pending U.S. Patent application Serial No. 245,351, filed September 16, 1988, which is a continuation of application Serial No. 906, 521, filed September 16, 1986, now U. S. Patent No. 4,795,698, which is a continuation-in-part of application Serial No. 784,863, filed October 4, 1985, now abandoned. The disclosure of the aforementioned related applications are incorporated herein by reference.

Field of the Invention

This invention pertains to stable suspensions of magnetic particles and to resuspendable coated magnetic particles, preferably with biochemical or biological activity, to compositions including such particles, and to methods of making and using such particles and compositions.

Biologically active magnetic particles find use in a variety of preparative and diagnostic techniques. Among these is high gradient magnetic separation (HGMS) which uses a magnetic field to separate magnetic particles from suspension. In instances where these particles are attached to biological materials of interest (e.g., cells, drugs), the material of interest

may thereby be separated from other materials not bound to the magnetic particles. Because of their magnetic properties, these materials also function as contrast agents for magnetic resonance imaging.

5

As used herein, the term "resuspendable coated particle" refers to a finely divided solid, which forms a colloidal suspension and may be separated from the suspension and subsequently resuspended. "Magnetic" encompasses material which may or may not be permanently magnetic, which also may be paramagnetic or superparamagnetic but which in all cases exhibits a response in a magnetic field, i.e., is magnetically responsive. "Disrupted" particles are those which are too small to contain a complete magnetic domain or, alternatively, whose Brownian energy exceeds their magnetic moment. Generally, these particles are less than 0.03μ in size.

20 Description of Related Art

Many techniques have been suggested in the prior art for the preparation of magnetic particles or organo magnetic materials. Such particles generally fall into three categories: large, small and microagglomerants of small particles. Large magnetic particles, having diameters greater than 10μ respond to weak magnetic fields and magnetic field gradients.

- 3 -

Because of their size, they tend to settle rapidly from solution and also have limited surface area per unit weight. Large particles also tend to aggregate after they have been subjected to a magnetic field because they can be permanently magnetized. Small particles which have magnetic cores of mean diameter less than 0.03μ remain in solution by virtue of their Brownian energy and hence do not spontaneously settle. Microagglomerants of such small magnetic particles have been prepared by various methods. Depending on the size of the microagglomerants, materials which can remain in solution for reasonable periods of time can be prepared. Additionally, the magnetic properties of small particles and microagglomerants of small magnetic particles are significantly different from those of the larger permanently magnetizable particles. Small magnetic particles composed of either single crystals of ferromagnetic materials such as iron oxides or agglomerants of such crystals become "super-paramagnetic" when the crystal size of the ferromagnetic materials is below about 300\AA . Unlike ferromagnetic crystals, superparamagnetic crystals only exhibit magnetic behavior when they are in a magnetic field gradient and do not become permanently magnetized. Such materials have been referred to as dispersible magnetic metal oxide particles and also as magnetically responsive particles.

- 4 -

One route to obtaining a magnetic particle bearing a bioreceptor is that of U.S. Patent Nos. 3,970,518 and 4,018,886 to Giaever, which teach the physical coating of such materials onto magnetic particles via adsorption. The coating of bovine serum albumin onto 1 μ diameter nickel particles is exemplified.

U.S. Patent No. 4,230,685 to Senyei et al. considers the teaching of U.S. Patent No. 3,970,518 and states that there is "no literature verification that uncoated magnetic particles can effectively be made to bind with antibody" and presumable other bioreceptors. U.S. Patent No. 4,554,088 to Whitehead et al. states that antibodies adsorbed on iron oxides are substantially detached by 24-hour 50° incubation in 1M sodium chloride and also that the quantity of adsorbed material is low.

With respect to one type of superparamagnetic particle described herein, namely colloidal particles, the method of recovery proposed in U.S. Patent Nos. 3,970,518 and 4,018,886 could not easily be made to work on such colloidal particles, as the field strength required to capture such particles for washing away unadsorbed materials would be enormous. Additionally, a field gradient is required, which is not achievable with the apparatus described. In conjunction with the

- 5 -

preparative use of high gradient magnetic separation (HGMS), the concept of Giaever might work where there are effective means for adsorbing on and retaining antibodies or bioreceptors to such particles.

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In view of the apparent failure to produce functionally acceptable magnetic particles via adsorption, a number of ingenious innovations have been reported. These include U.S. Patent No. 4,230,685 to
10 Senyei et al., which discloses the preparation of microspheres containing magnetite, albumin, and protein A. The preparation taught by Senyei involves an emulsion polymerization of the above ingredients. U.S. Patent No. 4,554,088 to Whitehead et al. teaches the silanation of
15 magnetic metal oxides which subsequently can be covalently linked to bioactive molecules. Both of the preceding teachings deal with agglomerated superparamagnetic particles; hence the agglomerated materials are classed as magnetically responsive. Other
20 patents which may be considered to be of interest include U.S. Patent No. 4,152,210 to Robinson et al.; 4,335,094 to Mosbach; 4,070,246 to Kennedy et al.; and 4,454,234 to Czerlinski. While these patents all disclose the preparation or use of magnetic-biologic particles, none
25 of these are thought to be similar to those of the present invention.

- 6 -

U.S. Patent No. 4,452,773 to Molday discloses "colloidal" iron oxide particles coated with non-ionic polysaccharide by forming magnetite in 25% (w/w) polysaccharide solutions. Molday further teaches the covalent linking of bioactive molecules to such formed particles by well-known chemical linking technology.

U.S. Patent No. 4,795,698 to Owen et.al, which is incorporated by reference herein, teaches the preparation of colloidal sized metal oxide particles which are coated in what is believed to be a covalent manner by polymers or proteins which have substantial numbers of unpaired electrons. Bioactive molecules such as antibodies or enzymes retain biological activity in the Owen et al. process which involves (1) the coprecipitation of transition element oxides and polymer or protein at 0.1 to 1 mg/ml by titration with base to slightly alkaline pH, (2) the subsequent washing of the coprecipitate and (3) the resuspension of the coprecipitate in appropriate buffers followed by mild sonication resulting in colloidal magnetically responsive particles. In that process, nearly all of the polymer or protein is precipitated. When dextran-coated particles were attempted in the process of Owen et al., a resuspendable colloidal particle could not be obtained. That result, along with the fact that Owen et al. requires coprecipitation with polymers having substantial numbers of unpaired electrons (in contrast to dextran and the

- 7 -

other nonreactive polysaccharides which Molday teaches) which apparently interact directly with transition metals, indicates that the processes of Molday and Owen et al. are substantially different. It should also be
5 noted that the Owen et al. process requires the protein or polymer to be in water or low ionic strength buffer.

In view of the apparent absence of interaction of ferric and ferrous ions with dextran and the nature of
10 the Molday dextran particle, it is instructive to examine the process taught therein. The colloidal particles of Molday are prepared by forming magnetite from ferric and ferrous chlorides with NH_4OH in the presence of 25% (w/w) aqueous Dextran T-20 (Pharmacia) or other similarly
15 concentrated polysaccharides. Aggregates formed in the process are subsequently removed by three cycles of centrifugation at low G force. Colloidal dextran magnetite in the supernatant is recovered following gel filtration which separates the unreacted dextran from the
20 colloidal particle which appears in the void volume. A substantial quantity of free dextran exists after the particles are formed. Although there is no discussion of the mechanism for colloidal dextran formation, it seems reasonable to suggest that dextran plays a physical or
25 "barrier" role in the process. The basis for this suggestion is that 25% (w/w) dextran solutions are extremely viscous with extensive hydrogen bonding due to

- 8 -

the interaction of its abundant hydroxyl groups with water. These factors create a system which limits diffusion. This would enable ferric and ferrous ions interacting with base to form local crystal nucleation sites whose growth will be related to the ability of bystander ions to participate. The presence of dextran could thus limit ionic participation resulting in the formation of small magnetite crystals (less than 300Å) which then are capable of adsorbing dextran molecules onto their surface. Thus, in this scenario, the dextran plays several roles in the process.

An alternative mechanism for formation of the Molday dextran magnetite is related to a fundamental property of magnetite. From the electron micrographs of Whitehead et al. and from the descriptive material of Senyei et al., it appears that magnetite prepared by the usual base precipitation of iron chlorides is composed of stable crystal of about 300Å or less in size. In view of the absence in the literature of reports indicating that magnetite can be made into a stable colloidal dispersion, it would seem that these crystals have a strong tendency to aggregate via mutually attractive molecular forces. On the other hand, by forming crystals "in situ" and in "chambers" formed by the high concentration of dextran employed by Molday and by having the "walls" of these individual chambers being able to collapse onto and coat such crystals, Molday appears to have been able to

achieve the production of a colloiddally stable magnetic particle. In view of the immense difference in surface areas provided by such individual magnetite crystals in contrast to the materials of Giaever, Molday appears to
5 have additionally serendipitously solved the adsorption problem for certain polysaccharides.

U.S. Patent No. 4,452,773 to Molday discloses a method for making colloidal iron oxide particles coated
10 with non-ionic polysaccharides by forming magnetite in 25% (w/w) polysaccharide solutions. Molday further teaches the covalent linking of bioactive molecules to such formed particles.

15 U.S. Patent No. 4,795,698 to Owen et al., which matured from a grandparent of this application and the disclosure of which is incorporated by reference here, teaches the preparation of colloidal sized resuspendable bioactive magnetic particles by the coprecipitation of
20 transition element oxides and either (a) a bioactive compound, such as a polymer or protein, or (b) a ligand for bioactive compounds.

The significance of the size of bioactive,
25 magnetically responsive particles, for NMR (or MRI) imaging, is disclosed, for example, in Renshaw, Owen,

- 10 -

Evans and Leigh, in Magnetic Resonance Imaging 351
(1986).

5 A colloidal dispersion of magnetic particles in
rocket fuel is disclosed in U.S. Patent 3,215,572 to
Papell. The dispersion is said to include magnetic
particles, such as magnetite (Fe_3O_4), 0.25μ in diameter
and smaller, preferably less than 0.10μ in diameter. The
10 dispersion is produced by ball milling a suspension of
larger particle size magnetic particles in the
propellant, with a grinding agent, which prevents
"agglomeration or welding of the minute particles as
grinding progresses." (column 2, lines 33-34) The ball
mill includes metal balls to perform the ball mill
15 action. The grinding agent, generally included at levels
on the order of 2% but possibly 10%, typically comprises
oleic acid; further "... other grinding agents such as
stearic acid and cetyl alcohol may be utilized in the
production of a magnetic propellant and other long chain
20 hydrocarbons having similar high surface tensions, such
as benzene, ethane, hydrazine and gasoline may be
utilized as the particle carrier and major constituent of
the magnetic propellant," (column 4, line 5-6).

Summary of the Invention

This invention relates to a new method for simplified production of magnetically responsive superparamagnetic particles. Magnetically responsive metal oxides can be coated effectively by employing in the coating process means for disrupting crystalline agglomerates such that coating can take place during the disruption. A wide range of materials (including dextran, proteins, synthetic polypeptides, polymers, copolymers, detergents and combinations thereof) can be coated onto such crystals resulting in colloidal magnetically responsive particles. In addition to obtaining colloidal materials, it is possible in most instances, by limiting the amount of coating material, to obtain stable microagglomerants which retain adsorbed material in an extremely effective fashion and which can be removed from solution with simple laboratory magnets.

Magnetic particles of small size (maximum particle size generally below 0.2μ) with a stabilizing (preferably biochemically or biologically active) coating, are produced, in accordance with the present invention, by forming a suspension of somewhat larger size parent magnetic particles (believed to be agglomerates), together with a material adapted to form a coating on subdivided "sub-particles" of the parent

- 12 -

particles. This mixture is then treated to subdivide or
disrupt the parent particles and to hold those particles
in that state while simultaneously permitting the coating
material to form a coating on the deagglomerated or
5 subdivided particles, thus stabilizing them at reduced
particle size. The product is a stable suspension.

With the proper selection of coating material,
the coated subdivided particle product can be separated
10 and resuspended. The resultant resuspendable product, if
the stabilizing coating is a bioactive compound or
ligand, is particularly useful as an MRI contrast agent,
for bioanalytical applications and for industrial
bioprocessing.

15 Preferably, the means for disrupting the parent
magnetic particle starting material in the suspension is
sonication, but other mechanical or chemical means may
also be used. These other "means" include, for example,
20 heating, other forms of particle energization, such as
irradiation, and chemical means, such as pH modification
or combinations of these types of treatment. In
particular, a combination of pH modification and
sonication may also be used.

- 13 -

Detailed Description of the Invention

5 It is hypothesized that magnetic compounds, in
particle form, tend to have significant surface polarity.
That net surface polarity is minimized by agglomeration
of crystals of such materials. When these crystals are
subdivided or disrupted, they tend to be unstable. In
10 accordance with the present invention, the nascent (and
probably charged) surfaces of these sub-particles are
stabilized by the coating material which is
simultaneously deposited on these surfaces as the parent
particles are sub-divided. For that purpose, the coating
15 material may be chosen with reference to its tendency to
respond to the surface polarity of the deagglomerated
magnetic particle and various coating materials will thus
react differently with different magnetic particle
materials. If the treating or disrupting technique is,
20 or includes, pH modification, the effect of pH
modification on sub-particle surface polarity and coating
material polarity may also be a consideration. The
coating material is selected in each case with regard to
its ability to adhere to or to be adsorbed on the
25 surface of the deagglomerated or sub-divided particle so
that the stability of the reduced particle size product
is retained, to form a stable suspension thereof.

This invention provides significant advantages over Molday and Owen et al. for preparing colloidal materials beyond the inherent simplicity of the method. For example, if it is desired to attach compounds to metal oxide particles where the compound has reactive groups or activated groups for subsequently attaching other materials (such as antibodies or enzymes), the Molday and Owen et al. procedures are limited by the choice of such compounds. This is because these processes necessarily start by mixing such compounds with metal chlorides which themselves can react with the compounds and which also create acidic pH. Further, base addition, usually ammonium hydroxide, is required to form the metal oxides and that can clearly have negative effects on a variety of activated chemical groups used for the subsequent coupling. By the direct adsorption methods of this invention, no such limitation exists. Further more, the coating of this invention can be done under non-aqueous conditions or where water is a minor component of the system. In some instances, coating materials can have limited aqueous solubility.

Certain types of coatings, such as the long chain hydrocarbons suggested in the Papell patent, have a detergent effect. These coatings stabilize the subdivided deagglomerated magnetic particles and produce a stable suspension, such as that taught in Papell. In

- 15 -

accordance with the present invention, these suspensions are produced, from magnetite and with sonication as the disrupting means, several orders of magnitude faster than in the ball milling process taught in Papell.

5

To produce resuspendable products, it is important to select a coating material which not only stabilizes the sub-divided magnetic particles, but does so with a coating which remains intact when the coated particles are removed from suspension, which is not the case with Papell.

10

Magnetic compounds which may be used as the starting material in the present invention include the transition metal oxides, sulfides, silicides and carbides, optionally having different transition metals in a single magnetic compound, such as $Gd_3Fe_5O_{12}$. Preferred are the class of magnetic oxides known as ferrites, generally represented as $MO \cdot Fe_2O_3$ in which M is Zn, Gd, V, Fe, Ni, Cu, Co, Mg, in particular magnetite ($FeO \cdot Fe_2O_3$).

15

20

In addition to the transition elements taught by Owen et al. and the ferrites, above, a class of magnetic metal oxide which does not contain iron can be coated as described in this invention. These compounds include oxides of combinations of 2 or more of the

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- 16 -

following metal ions: Al(+3), Ti(+4), V(+3), Mb(+2),
Co(+2), Ni(+2), Mo(+5), Pd(+3), Ag(+1), Cd(+2), Gd(+3),
Tb(+3), Dy(+3), Er(+3), Tm(+3) and Hg(+1). They differ
from ferrites in both appearance and magnetic
5 susceptibility. The non-ferrites can take any color from
white or yellow to green and even brown. This makes them
particularly useful in spectrophotometric applications.
Non-ferrites are generally less strongly magnetic than
ferrites and, as such, pass through HGMS filters in
10 magnetic fields capable of collecting ferrite based
materials which permits selective magnetic retrieval.

The non-ferrous oxides can be employed in place
of the metal oxides described by Whitehead et al. to
15 produce silane coated magnetic particles which have the
desirable properties given above. Similarly, when the
chlorides (or sulfates) of such combinations are employed
with the methods taught by Molday or by Owen et al.,
coated product having very desirable magnetic and
20 spectral properties can be obtained.

As indicated above, coating materials which may
be used are preferably in aqueous suspension or solution,
although suitable coating materials in non-aqueous
25 solvents or as melts may also be used. The coating
material is usually a synthetic or natural polymer and
may be a protein or a nucleic acid.

- 17 -

These materials are combined in a liquid mixture, usually including a third component such as water, to form a suspension. The relative proportions of these materials in this mixture is not critical. However, in general, the proportion of magnetic particles to coating material is from 1000:1 to 1:10 (by weight).

To make stable suspensions of coated sub-particles of the magnetic material, the mixture may be treated in a number of ways to disrupt or sub-divide the magnetic particulate starting material. These include mechanical and chemical means, such as heat, vibration, irradiation, sonication, pH modification, or a combination of these. Of these, sonication is much preferred.

We have also discovered that the employment of high gradient magnetic separation (HGMS) in the production of such particles gives a dimension to their production which heretofore has not been achieved or recognized. The Molday and Owen et al. processes utilize either centrifugation, gel filtration or "salting out" for manipulative procedures during the processing of colloidal materials. In the example herein, HGMS has been used to separate colloidal particles from unbound coating substance. In processes where it might be

- 18 -

desirable to chemically couple substances to such particles, HGMS offers process facilitation which is readily scaled and highly efficient.

5 HGMS can also be used in concert with tuned magnetic fields to fractionate preparations based on their magnetic susceptibility/particle volume ratios. This permits convenient fractionation and production of particle preparations of discrete sizes. When used as
10 NMR contrast agents, the size of the particle plays an important role in the metabolic fate of the material. HGMS, again with tuned magnetic fields, can also be used to selectively capture particles where the magnetically responsive core contains transition element oxides having
15 higher magnetic susceptibilities than others in the mixture. This concept could have utility in a system whereby colloidal particles having different magnetic susceptibilities as well as different bioreceptors could be mixed with a sample and removed by sequential HGMS
20 using increasing gradient field strength.

 Not only can HGMS be used to remove unadsorbed coating or unreacted substances and by-products from colloidal particles, it can also be utilized for
25 immobilizing coated magnetic products and doing reactions on the immobilized material. Thus, if it is desired to

- 19 -

chemically modify a given coating, reactants can be added to magnetically immobilized material, the reaction performed in that state and excess reactant or other reaction products easily washed away. This concept, somewhat like peptide synthesis done on solid supports, lends itself nicely to sequential reactions.

The following examples will serve to illustrate the principles of this invention; but the range of these examples should not be construed as limiting the scope of this invention.

Example 1

All reagents and chemicals used in these experiments were of analytical grade and obtained from Fisher Scientific (Valley Forge, PA) unless otherwise specified. Magnetite was prepared by mixing solutions of 3.0 and 1.5 mg/ml of ferric chloride hexahydrate and ferrous chloride tetrahydrate, respectively, with stirring at room temperature while raising the pH to 8.6 with NH_4OH . The resultant magnetite was collected magnetically, washed 3 times with distilled water and resuspended in distilled water. The preparation so made contained 1.5 mg/ml magnetite. Even after sonication (Fisher - Sonic Dismembrator Model 300) for 3 minutes at

- 20 -

70% output, these preparations will not remain suspended for more than about 2 minutes.

To coat magnetite particles by the method of this invention 0.5 ml aliquots of various concentrations of various coating materials were mixed with 0.5 ml aliquots of the 1.5 mg/ml magnetite suspension. Samples were mixed in conical plastic centrifuge tubes and subsequently sonicated for 3 minutes at 70% output at room temperature. A positive or partially positive coating result was apparent from visual inspection of the manner in which light was scattered by the sample.

To determine the efficiency of coating, resultant samples were further observed for settling and also were fractionated into colloidal coated magnetite or microagglomerants thereof in the following fashion: 0.5 ml aliquots of the sonicated mixture in 12 x 75 mm test tubes were placed in a Ciba-Corning Magnetic Separator (Walpole, MA) and visually observed. The criterion used to determine if the coated magnetite crystals were colloidal was to observe if the resultant material remained in solution in the magnetic separator for a period of 10 minutes, i.e. in the magnetic supernatant. This criterion was established on the basis that solutions of protein coated colloidal magnetite prepared by the process of Owen et al. could not be separated

- 21 -

magnetically in the Magnetic Separator when placed therein for such periods.

5 To determine what portion of the sonicated
mixtures formed stable, coated, but agglomerated
materials which pulled to the sides of the test tubes in
the Magnetic Separator, materials so formed were washed
with 20 mM phosphate three times and resuspended in the
same buffer. Their resuspension characteristics compared
10 with uncoated magnetite were distinctly different, as
they would remain suspended for hours compared with
minutes for the latter.

15 A second criterion involved examining the
magnetic supernatant as follows: Putative colloidal
material was separated from the mother liquor by HGMS,
washed, and resuspended in buffer, and visually observed
for colloidal appearance and stability. HGMS was
accomplished by placing approximately 20 mg of fine grade
20 stainless steel wool (McMaster-Carr, New Brunswick, NJ)
(washed in detergent, incubated in 1% BSA phosphate
buffered saline (PBS), rinsed with deionized water, dried
and cut in approximately 3 mm lengths) into 12 x 75 mm
glass test tubes. 100 microliters of the supernatants to
25 be tested were added to the test tubes containing the
stainless steel wool and placed in the magnetic
separating rack for 2 minutes by which time magnetic
material had collected on the stainless steel wires.

- 22 -

Clear non-magnetic supernatants were removed with Pasteur capillary pipettes and the magnetic material washed 3 times with 300 microliters of 20 mM phosphate (pH 7.5). After the 3rd wash, collected magnetic material was
5 resuspended in the phosphate buffer, after removing the tube from the magnetic rack, and examined visually for the appearance of a stable colloid.

In some instances recovered material was also
10 sized by laser light scattering (Coulter Sub Micron Particle Analyzer N4SD, Hialeah, FL). Some supernatants were separated from unadsorbed material by gel filtration chromatography on Sephacryl-300 (Pharmacia) or ultra gel ACA-22.

15
Table I lists different compounds used in coating experiments, their concentration(s), and other solvent conditions. The presence and semi-quantitation of colloidal and agglomerated materials were determined
20 visually by the procedures described above, by comparison to standards prepared from known mixes of colloidal and agglomerated stocks. As can be seen from the Table, every compound tested produced some amount of stable colloidal material. Where noted, stability, following
25 HGMS and resuspension in phosphate buffer, is also indicated. In all cases the success of the coating experiment could be determined immediately following the

- 23 -

sonication procedure from light scattered or by the
apparent "shininess" of the solution. Materials for
which coating conditions were inappropriate (i.e., stable
coated subdivided particles were not produced) were by
5 contrast dull and heterogeneous in appearance.

- 24 -

TABLE I

5	Supernatant Coating Material and Concentration (wt %)	Percent Colloidal	HGMS Recovery	Treatment of Colloidal Subsequent Resuspension
	Dextran, 25%	100%	Good	Good
10	Dextran, 12.5%	50%	Good	Good
	Dextran, 6.25%	25%	ND	ND
	Dextran, 3.12%	5%	ND	ND
	SDS, .5%	5%	ND	ND
15	SDS, .25%	20%	ND	ND
	SDS, .125%	80%	Good	Poor
	SDS, .063%	50%	ND	ND
	Tween-20, 1%	20%	Good	Fair
20	Tween-20, .5%	30%	ND	ND
	Tween-20, .25%	40%	ND	ND
	Tween-20, .125%	50%	ND	ND
	Tween-20, .063%	75%	ND	ND
	Tween-20, .031%	90%	ND	ND
25	Tween-20, 3.5%	50%	ND	ND

- 25 -

	BSA, 1% ; 50mM P	95%	Good	Good
	BSA, 7.5%; 50mM P	80%	ND	ND
	BSA, 7.5%; 40mM P	75%	ND	ND
5	BSA, 7.5%; 30mM P	50%	ND	ND
	BSA, 7.5%; 20mM P	90%	ND	ND
	BSA, .25%; .016% Tween-20	95%	Good	Good
10	IgG(human), 5%; PBS, 50%	60%	Good	Good
	Lipid Stripped Human Sera	100%	Good	Good
15	G α MFC/50mM P 1:2	5%	ND	ND
	G α MFC/50mM P 1:4	50%	ND	ND
	G α MFC/50mM P 1:8	5%	ND	ND
	G α MFC/50mM P 1:16	30%	ND	ND
20	G α MFC/50mM P 1:32	20%	ND	ND
	G α MFC/50mM P 1:64	5%	ND	ND
	Steroid Free, Lipid Stripped Sera, Neat	5%	ND	ND
25				

- 26 -

	Steroid Free, Lipid Stripped Sera/50mM P (1:128)			
		50%	ND	ND
5	PEG, 20%	80%	ND	ND
	GLA, 10%	100%	ND	ND
	poly G, 10%	15%	ND	ND
10	polyvinyl pyrrolidone, 15%	75%	ND	ND
	polyvinyl alcohol, 10%	50%	ND	ND
15				
	ND	= Not Done		
	P	= Phosphate buffer		
20				
	G α MFc	= goat anti-mouse Fc [Jackson Labs, West Grove, PA]		
	SDS	= sodium dodecyl sulfate		
25				
	PEG	= polyethylene glycol [Matheson, Coleman and Bell, East Rutherford, NJ]		

- 27 -

Dextran = Dextran T-40 [Pharmacia, Piscataway, NJ]

poly G = polyglutamic acid [NEN, Pilot Chemical
Division, Boston, MA]

5

GLA = [(glutamic acid 45 mole%) (lysine
35 mole%) (alanine 20 mole%)]_n
[NEN, Pilot Chemical Division, Boston, MA]

10 IgG = Immunoglobulin G

Lipid stripped and steroid free lipid stripped sera
from Scantibodies Inc., Santee, CA

15 Tween-20 = Polyoxyethylene surfactant (ICI
Americas)

Example 2 Quantitation of Protein Coating and
Retention

20

I-125 labeled BSA and IgG were prepared by the
Iodogen method of Fracker and Speck (Biochem. Biophys.
Res. Comm. 80 849, (1978)). Specific activities were
520,000 cpm/ug and 810,000 cpm/ug, respectively.

25

- 28 -

BSA was coated onto magnetite under two sets of conditions as follows (1) at 7.5% BSA in 10 mM phosphate buffer followed by 3 minute sonication and (2) at 7.5% BSA in 25 mM phosphate buffer followed by 3 minute sonication. In both cases 800,000 cpm of labeled BSA was added to the mixture (0.5 ml) prior to sonication. From visual inspection it was apparent that the higher concentration of phosphate resulted in significantly more colloidal material. The magnetically collectable precipitate for each experiment was collected, washed twice in 20 mM phosphate buffer and counted. The magnetic agglomerated material of conditions 1 and 2 contained 2421 and 2828 cpm, respectively. To determine how well BSA had adsorbed to these agglomerated preparations the recovered material was suspended in 0.1 M glycine at pH 3.0 for 40 minutes at room temperature, magnetically separated and washed once with 20 mM phosphate buffer. For experimental conditions 1 and 2, counts retained were respectively 91 and 84%. When similar preparations were resuspended in buffer and allowed to incubate over night at 37°C, no material desorbed from the magnetite.

To determine the stability of the colloidal magnetite BSA, the amount of BSA adsorbed onto the colloid was quantified as follows: 3x100 ul aliquots of the supernatant were individually collected by HGMS,

- 29 -

washed 2 times with 300 ul of phosphate buffer and resuspended into 100 ul of the same buffer. By observation over time (16 hours) it was apparent that the colloidal material was stable. Radioactive BSA recovered by HGMS for the total supernatant was 5400 cpm. From radioactivities and volumes of the supernatant and agglomerated materials obtained for condition 2 it was determined that 7,828 cpm's had been incorporated onto magnetite. This amounts to 0.01% of the total BSA added to the system which corresponds to about 0.75 mg/ml of magnetite so prepared. This value is very close to the optimal amount of BSA which can be coated onto magnetite by the procedure of Owen et al.

The IgG coating experiments were done at 5% protein concentration in PBS/2 (a 1:2 dilution of PBS in H₂O). Before sonication, mixtures were spiked with 1.8×10^6 cpm of radio labeled IgG. For these experiments it was found that 1.2% of the total added protein was adsorbed which corresponds to 1.2mgs IgG/ml of magnetite. Again this is close to the maximum coating obtainable by the Owen et.al. teaching. When HGMS was performed on the magnetic supernatant of this experiment 23,000 cpm were retained on the colloidal material. The agglomerated magnetic pellet retained 17,300 cpm's after multiple washings. When the magnetic pellet was resuspended in glycine at pH 3.0 as above only 50% of the counts were retained on the subsequent magnetic pellet.

- 30. -

However, in these experiments it was found that glycine treatment of the microagglomerated material had converted a substantial portion (approximately half) to colloidal material; hence, the IgG coated material is indeed
5 stable. Colloidal materials recovered by HGMS were stable by visual observation.

Example 3 Retention of Biological Activity

10 Goat anti-mouse Fc (obtained from Jackson Laboratories, West Grove, PA) was coated onto magnetite as described in Example 1. For coating, the untreated anti-sera was diluted 1:4 with 50 mM phosphate. After
15 the sonication procedure, most of the resultant material appeared colloidal. The entire supernatant was separated on Sephacryl-300. Colloidal material appearing in the void volume was recovered and the following test performed: 100 ul of the recovered colloid was mixed
20 with either 100,000 counts of 125-I labeled mouse IgG or 100,000 counts of 125-I BSA. These mixtures were incubated in 12 x 75 mm tubes in the presence of iron powder as described above. After 90 minutes at room
25 temperature, HGMS was performed, as described above, supernatants were discarded, and collected material washed twice with 0.8 mls of PBS containing 2% BSA. For these colloidal samples (in triplicate) it was found

- 31 -

that, on average, 5,200 counts of mouse IgG was bound to the Fc-coated colloid versus 632 cpm of nonspecifically bound BSA.

5 Example 4 Light Colored Particles

 Mixed transition metal oxides were prepared at room temperature and at 65°C by addition of base to appropriate metal chlorides as described in Example 1.
10 Table II lists preparative conditions, molar ratios, initial color of the oxides and color after 1 week, or color after bubbling O₂ through freshly prepared oxides for eight hours.

TABLE IIConcentration (mMole/Liter)

5

	Part.	FeCl ₂	VCl ₃	CoCl ₂	PdCl ₃	DyCl ₃	ErCl ₃	Original Color	Final Color*
	1	---	5	---	---	5	---	dk green	white
10	2	2.7	3.6	---	---	3.6	---	dk green	yellow
	3	5	3.3	---	---	1.7	---	dk green	orange
	4	6	---	---	---	4	---	dk green	rust
	5	---	---	5	5	---	---	green	brown
	6	---	---	---	5	5	---	brown	brown
15	7	---	5	5	---	---	---	blue/green	green
	8	---	5	---	---	---	5	lt brown	white
	9	---	---	---	5	---	5	brown	brown
	10	---	---	---	---	5	5	white	white
	11	5	---	---	---	---	5	green	orange

20

* These oxides change color gradually in about one week, or in about eight hours if the new precipitate is bubbled through with oxygen.

- 33 -

Table III lists the results of coating the oxide preparations of Table II (preps 1, 2, 3 and 4) with dextran and with BSA, according to the method of this invention. Clearly these materials can be coated similarly to magnetite. It is noted that the non-ferrite and ferrite oxides listed in Table II could be used in place of magnetite with the teaching of Whitehead et al. to produce similarly colored silane coated materials. When the appropriate chlorides (or sulfates) of these metals are used with the teachings of Molday or with Owen et al., it is possible to obtain colloidal materials which are nearly transparent in the visible region.

- 34 -

TABLE III

<u>Appearance After Sonication</u>					
5	<u>Particle</u>	<u>Compound</u>	<u>Supernatant</u>	<u>Pellet</u>	<u>% Colloidal</u>
	1	25% Dextran	Shiny White	White	90%
	2	25% Dextran	Cloudy Yellow	White	20%
	3	25% Dextran	Clear Yellow	White	95%
10	4	25% Dextran	Clear Orange	Orange	50%
<hr/>					
	1	1% BSA/50mM P	Shiny White	White	90%
	2	1% BSA/50mM P	Cloudy Yellow	White	90%
15	3	1% BSA/50mM P	Clear Yellow	White	95%
	4	1% BSA/50mM P	Clear Orange	Orange	75%

- 35 -

Example 5 Colloid Particle Size

BSA magnetite was prepared with the magnetite of Example 1 and BSA at 1% in 50mM Phosphate at pH 7.0 as described above except that an aliquot was sonicated for a second three minute period and another for a third three minute period. After removal of any agglomerated material with the magnetic rack, as described above, the resultant colloidal samples were sized by laser light scattering (Coulter Submicron Particle Analyzer). Within experimental error each preparation had a mean particle diameter of 80nm.

Next, magnetite for coating was prepared by two methods which have been found to decrease particle size in the Owen et al. procedure, namely very rapid addition of base and very rapid addition of base at elevated temperature (65°C). When BSA magnetite was prepared with a single 3 minute sonication step but with magnetite prepared according to Owen et al., the resultant colloids were both of 50nm mean diameter.

Example 6 Properties of Magnetite as
Affected by pH

25

3.75% NH_4OH was added at 0.6 mls/minute to a 200 ml stirred mixture of degassed hydrated Ferric and Ferrous chloride salts at 7 and 3 mg/ml respectively.

- 36 -

As the pH of the initially acidic mixture approached 7.0 (indicted by a color transition from dark orange to black), 300ul aliquots were removed, placed in 10x75 glass test tubes, and tested for the presence of magnetite, by placing a neodymium/iron/boron bar magnet to the side of the tube and visually observing magnetic clearing. The transition from non-magnetic or partially magnetic material to totally magnetic material occurred at pH 7.4 after 12.2 mls of base had been added. At this point, 60 ml of the mixture was removed and designated preparation A. Additional base was added to the mixture to pH 8.9; a 60 ml aliquot was removed and labelled preparation B. Base was further added to the reaction mixture to pH 9.8 and an aliquot of this material designated preparation C. Magnetite preparations A, B, and C were washed 4 times with distilled water and resuspended in water such that the iron salts were at the starting concentrations of the original solution. 0.5 ml aliquots of the latter suspensions of A, B and C were sonicated for 3 minutes at 70% output (Fisher Sonic Dismembrator). Immediately following sonication, each had the shiny appearance of colloidal magnetite; however, all reverted to dull suspensions (indicative of particle aggregates) within 2 minutes. For preparation C, the transition occurred 20-30 seconds following sonication; for preparation B, 40-50 seconds; while preparation A required 2 minutes for this transition. When the preparations were placed

in the Corning magnetic rack immediately following sonication, they cleared completely from suspension in the same order as above and over the same time frame. After reaggregation, there was no discernible
5 difference among the samples.

To determine if prepared magnetites' susceptibility to dissociation by sonication is altered by H^+ or OH^- treatment, 0.5 ml aliquots of the
10 preparation C suspension were placed in test tubes and separated from their water supernatants by magnetic separation and supernatant aspiration. Magnetic pellets were then resuspended into 0.5 ml of either dilute HCl (0.1, 0.01, 0.001, or 0.0001 M) or dilute
15 NaOH (0.1, 0.01, 0.001, or 0.0001 M), sonicated for 3 minutes as above and visually inspected. For these samples, it was apparent that acid or base treatment increased the disruption of magnetite in proportion to concentration of acid or base. The aliquots
20 resuspended and sonicated in 0.1 M HCl or NaOH remained shiny, i.e. colloidal, for nearly 12 hours. The aliquots resuspended in 0.001 M acid or base also exhibited colloidal behavior for significant periods of time. On the other hand, aliquots resuspended into
25 0.0001 M acid or base quickly aggregated after sonication much like the parent material did in water.

Example 7 Coating of pH Modified Magnetite

Preparation C of Example 6 was coated by disruption via sonication with anionic and cationic polypeptides. An anionic terpolypeptide composed of 60 mole % glutamic acid, 30 mole % alanine, 10 mole % tyrosine (GAT, Lot #M18G) and a cationic copolymer of 60 mole % lysine, 40 mole % alanine (LA, Lot #M-5B), both obtained from Pilot Chemicals, Watertown, MA, were used. These polypeptides, both of about 100,000 Daltons, were solubilized by tituration with appropriate acid or base, neutralized, dialyzed versus phosphate buffered saline (pH 7.0) and subsequently against distilled water. Coatings were attempted with 10 mg per/ml solutions of polypeptide and serial dilution of same to 1/16. Coating experiments were done by sonication as described above. For these experiments, the 1/16 and 1/8 dilutions of both GAT and LA produced stable colloidal solutions. The 1/4, 1/2 and undiluted polypeptide solutions resulted in distributions of colloid and agglomerated material. The amount of colloid in both instances generally decreased with increasing polypeptide concentration. However, at 25 mg/ml GAT all of the material was colloidal.

Preparations A and C of Example 6 were coated with GAT. 0.5 ml aliquots of magnetite were resuspended in 1.0 ml of HCL (0.01 and 0.1 M) or NaOH (0.01 and 0.1M) for 60 seconds followed by two 0.5 ml water washes. These pre-treated magnetites were resuspended in 0.5 ml aliquots of 1 mg/ml GAT containing 2.5×10^6 cpm ^{125}I GAT (radio-labeled by the Iodogen method described above) and sonicated as described. Sonicated samples were placed in the Corning magnetic rack and left to separate overnight. Magnetic pellets were washed once in 0.5. M NaCL and counted. Counts bound and percentages of GAT coated onto the various magnetites are given in Table IV.

TABLE IV

Effect of H^+/OH^- Treatment of Magnetite
Preparations A and C on GAT Coating

	<u>Treatment</u>	<u>Preparation A</u>		<u>Preparation C</u>	
		<u>cpm bound</u>	<u>% bound</u>	<u>cpm bound</u>	<u>% bound</u>
	0.10 M HCL	450,000	35	340,950	26
10	0.01 M HCL	287,602	22	226,250	17
	H ₂ O Control	209,165	16	236,042	18
	0.01 M NaOH	130,728	10	N.D.	N.D.
	0.010 M NaOH	90,600	8	54,010	5
15	N.D. = Not done				

From the data of Table IV, it is clear that magnetite crystal surface charge can be made more positive by a pre-treatment with acid and conversely, treatment with base generally reduces sites available to the negatively charged polymer. When ^{125}I preparation of the cationic terpolymer LAT (60 mole % lys, 30 mole % ala, 10 mole % tyr), (received from Dr. H. J. Callahan, Jefferson Medical College, Philadelphia, Pa.) was used with similarly pre-treated magnetite, it was observed that greater binding of this positively charged material occurred on magnetite pre-treated with base.

To determine if crystal surface charged alteration via acid or base treatment is a general property of transition element oxides, such as those prepared from different molar ratios of chlorides of Fe (II), Dy (III), V (III), such materials were treated with acid or base as above and mixed with either radio-labeled GAT and/or Salmon sperm DNA (Sigma Chemical Co., St. Louis, MO). As in the case with magnetite, acid treatment promoted binding of these negatively charged polyelectrolytes. In fact, uncoated materials pretreated with 0.1 M HCl, followed by water wash and sonication give materials which behaved like preparations of magnetite described above, i.e., transiently stable colloids. When these materials were in this semi-stable colloid state, addition of Salmon sperm DNA in appropriate quantities lead to complete agglutination indicating the crystals had assumed substantial positive surface charge.

We claim:

5 1. A process for making resuspendable coated magnetic particles, said process comprising:

10 a. forming a liquid mixture of a particulate magnetic starting material and a coating material characterized by the ability to form a coating on disrupted particles of said magnetic starting material,

 b. treating said mixture to subdivide the particles of said magnetic starting material,

15 c. simultaneously with step b., permitting said coating material to form a coating on the subdivided particles of said magnetic starting material to form stable, resuspendable coated particles of said magnetic starting material,

20 d. recovering the resuspendable coated magnetic particles from the liquid mixture.

25 2. A process, as recited in claim 1, wherein the liquid mixture is an aqueous suspension.

3. A process, as recited in claim 1, wherein the magnetic starting material is a transition metal oxide.

5 4. A process, as recited in claim 3, wherein the transition metal oxide is magnetite.

5. A process, as recited in claim 1, wherein the coating material is a natural or synthetic polymer.
10

6. A process, as recited in claim 1, wherein the coating material is a polypeptide, a protein, or an antibody.

15 7. A process, as recited in claim 1, wherein said mixture is treated by irradiation, vibration, pH modification, or sonication.

20 8. A process, as recited in claim 1, wherein said mixture is treated by sonication.

9. A process, as recited in claim 1, wherein said coated particles generally have a maximum dimension of less than 0.2μ .
25

10. A process, as recited in claim 1, wherein said coated particles generally have a maximum dimension of less than 0.1μ .

11. A process, as recited in claim 1, wherein the weight ratio of magnetic material to coating material is from about 1000:1 to about 1:10.

5

12. A process, as recited in claim 1, in which the resuspendable coated particles are recovered by high gradient magnetic separation.

10

13. A process, as recited in claim 1, comprising recovering the magnetic coated particles by fractionating the suspension of step c) into colloidal magnetic coated particles, microagglomerants of coated magnetic particles, and unreacted coating and magnetic particles.

15

14. A process, as recited in claim 13, in which the fractionation is by high gradient magnetic separation.

20

15. A process, as recited in claim 1, further comprising resuspending the magnetic coated particles in water or an aqueous buffer solution.

25

16. A process, as recited in claim 15, wherein said resuspension is conducted under the influence of sonication.

17. A process, as recited in claim 1, further including the step of reacting said magnetic coated particles with a bifunctional compound specific to the coating.

5

18. A process, as recited in claim 1, further including the step of reacting said magnetic coated particles with a bifunctional compound adapted to form primarily intraparticulate bonds.

10

19. A process, as recited in claim 1, further including the step of reacting said magnetic coated particles with a bifunctional compound and reacting the product particle with a bifunctional ligand.

15

20. A process, as recited in claim 1, further including the step of reacting said magnetic coated particles with an activating agent and reacting the product particles with a bifunctional ligand.

20

21. A process for making a stable suspension of resuspendable magnetic particles, said process comprising:

25

a. forming a liquid mixture of a particulate magnetic starting material and a coating material characterized by the ability to stabilize disrupted particles of said magnetic starting material,

b. sonicating said mixture to disrupt the particles of said magnetic starting material and,

5 c. simultaneously with step b.,
permitting said coating material to form a coating on the
disrupted particles of said magnetic starting material,
forming a stable suspension thereof.

10 22. Magnetically responsive coated particles
produced by the process of claim 1.

23. A process for fractionating a suspension
of magnetically responsive coated microparticles
15 comprising high gradient magnetic separation of the
suspension.

24. A process of claim 23 further comprising
the application of tuned magnetic fields.

20 25. A magnetically responsive coated
microparticle having a non-ferrous superparamagnetic core
and a polymeric coating.

25 26. A microparticle of claim 25 in which the
polymeric coating is a polysaccharide or a derivative
thereof.

- 47 -

27. A magnetically responsive coated microparticle in which the core comprises oxides of at least two different transition metals and the resultant coated particle is colored white at equilibrium.

5

28. A microparticle of claim 27 in which the transition metals are selected from Dy, Er and V.

29. A microparticle of claim 27 comprising a core of mixed oxides selected from Dy and V oxides, V and Er oxides, and Dy and Er oxides.

10

30. A superparamagnetically responsive coated microparticle having a mean diameter of from about 5 to about 500 nanometers, comprising a partially ionized magnetite core and a polymeric coating of opposite charge adsorbed to said core.

15

31. A method for reacting a coated magnetic particle with a suitable reactant comprising immobilizing the magnetic particle in a high gradient magnetic separator, adding the desired reactant, maintaining said reactant and said particle in proximity, and washing out the reaction products.

20

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04459

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/10 ; G01N 33/53; C12N 11/06; G01N 33/553; B32B 5/16; A01N 1/02; C01G 49/08; B01D 35/06 U.S.C.: 435/4; 210/222; 252/62.56; 427/2; 428/402; 435/7; 435/181; 436/526																				
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.C1.</td> <td style="padding: 5px;">435/4; 210/222; 252/62.56; 427/2 428/402; 435/7; 435/181; 436/526; 436/533</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵</div> <p style="padding: 10px 0 0 40px;">APS, CAS, BIOSYS</p>			Classification System	Classification Symbols	U.S.C1.	435/4; 210/222; 252/62.56; 427/2 428/402; 435/7; 435/181; 436/526; 436/533														
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category [*]</th> <th style="width: 60%; padding: 5px;">Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷</th> <th style="width: 30%; padding: 5px;">Relevant to Claim No. ¹⁸</th> </tr> </thead> <tbody> <tr> <td style="vertical-align: top; padding: 5px;"><u>X</u> Y</td> <td style="padding: 5px;">US, A, 4,795,698 (Owen et al.) 03 January 1989, see the entire document.</td> <td style="padding: 5px;">1-10, 15-22, 25-27 11-14, 23, 24, 28-31</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;"><u>X</u> Y</td> <td style="padding: 5px;">US, A, 4,452,773 (Molday) 05 June 1984, see the entire document.</td> <td style="padding: 5px;">1-7, 9, 10, 15, 17, 18, 25-26 8, 11-14, 16, 19 20-24, 27-31</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;"><u>X</u> Y</td> <td style="padding: 5px;">US, A, 4,554,088 (Whitehead et al.) 19 November 1985, see entire document.</td> <td style="padding: 5px;">1-4, 22 5-7, 9-15, 20</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;"><u>X</u> Y</td> <td style="padding: 5px;">US, A, 4,628,037 (Chagnon et al.) 09 December 1986, see entire document.</td> <td style="padding: 5px;">1-4, 22 5-7, 9-15, 20</td> </tr> <tr> <td style="vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 3,907,518 (Giaever) 20 July 1976, see abstract.</td> <td style="padding: 5px;">1-21</td> </tr> </tbody> </table>			Category [*]	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	<u>X</u> Y	US, A, 4,795,698 (Owen et al.) 03 January 1989, see the entire document.	1-10, 15-22, 25-27 11-14, 23, 24, 28-31	<u>X</u> Y	US, A, 4,452,773 (Molday) 05 June 1984, see the entire document.	1-7, 9, 10, 15, 17, 18, 25-26 8, 11-14, 16, 19 20-24, 27-31	<u>X</u> Y	US, A, 4,554,088 (Whitehead et al.) 19 November 1985, see entire document.	1-4, 22 5-7, 9-15, 20	<u>X</u> Y	US, A, 4,628,037 (Chagnon et al.) 09 December 1986, see entire document.	1-4, 22 5-7, 9-15, 20	A	US, A, 3,907,518 (Giaever) 20 July 1976, see abstract.	1-21
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>																				
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px; vertical-align: top;"> Date of the Actual Completion of the International Search ² <div style="text-align: center; padding: 5px;">27 November 1990</div> </td> <td style="width: 50%; padding: 5px; vertical-align: top;"> Date of Mailing of this International Search Report ³ <div style="text-align: center; padding: 5px; font-size: 1.2em;">18 JAN 1991</div> </td> </tr> <tr> <td style="padding: 5px; vertical-align: top;"> International Searching Authority ¹ <div style="text-align: center; padding: 5px;">ISA/US</div> </td> <td style="padding: 5px; vertical-align: top;"> Signature of Authorized Officer ¹⁰ <div style="text-align: center; padding: 5px;"> SCOTT A. CHAMBERS </div> </td> </tr> </table>			Date of the Actual Completion of the International Search ² <div style="text-align: center; padding: 5px;">27 November 1990</div>	Date of Mailing of this International Search Report ³ <div style="text-align: center; padding: 5px; font-size: 1.2em;">18 JAN 1991</div>	International Searching Authority ¹ <div style="text-align: center; padding: 5px;">ISA/US</div>	Signature of Authorized Officer ¹⁰ <div style="text-align: center; padding: 5px;"> SCOTT A. CHAMBERS </div>														
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Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹ not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-22 drawn to a process of making magnetic particles and the particle made.
- II. Claims 25-30 drawn to other microparticles with polymeric coating and oxide core as well as claims 23 & 24 drawn to a process of fractionating.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Attachment To Form PCT Telephone Memorandum
Continuation

I. Claims 1-22 drawn to a first process for making magnetic particles and first product made.

II. Claims 22 and 25-30 drawn to other microparticles with polymeric coating and oxide core.

Claims 23 and 24 drawn to a second process of fractionating.

There is no provision in PCT Rule 13.2 for multiple products or methods of making.